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Effects of Hypoxia on Granulocytic-Monocytic Progenitors in Rats. Role of Bone Marrow Stroma

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Hemorrhagic shock leads to hypoxia and is associated with bone marrow (BM) failure. Hemorrhagic shock is also a predisposing factor in immune dysregulation. Since the BM is the major organ of immune cells in the adult, its failure following hemorrhagic shock may explain the increased susceptibility to infection. The in vitro evidence indicates that hypoxia mediates altered functions in BM stroma. Since similar hematopoietic alterations are reported in hypoxia and hemorrhagic shock, hypoxia alone could be a representative model to study BM responses during hemorrhagic shock. In this study, we use an animal model to dissect the hematopoietic effects of hypoxia. We subjected rats to hypoxia, and at days 1 and 5 post-hypoxia we determined the numbers of granulocytic–monocytic progenitors (CFU-GM) in the BM. We found significant increase ($P < 0.05$) in CFU-GM at day 1 and a downward trend by day 5. Enhanced BM cellularity could not explain the increase in CFU-GM by day 1. BM stromal cells mediated most of the stimulatory effects by hypoxia. CFU-GM was inversely proportional to bioactive TGF-β and directly proportional to IL-1. Compared to normoxic rats, IL-6 production was suppressed in BM cells from hypoxic rats. The results show that hypoxia alone initiate a stimulatory response in CFU-GM progenitors. These effects are at least partially mediated through the BM stroma. In the absence of a second insult, CFU-GM reverts to baseline. The data also suggest that hypoxia mediates complex responses that include cytokine production. These results add to the current understanding of hematopoietic responses by hypoxia and adds to the mechanisms of immune dysfunctions following hemorrhagic shock. Am. J. Hematol. 64:20–25, 2000.

Key words: bone marrow; hypoxia; hematopoiesis; hemorrhagic shock; transforming growth factor-β

INTRODUCTION

Hemorrhagic shock is a significant predisposing factor in the development of adult respiratory distress syndrome and organ failure following trauma [1–4]. The experimental evidence indicates that hemorrhagic shock is associated with altered immune and hematopoietic functions [5–9]. Immunosuppression, associated with hemorrhagic shock, appears to be reversible [10–12].

Regional and cellular hypoxia, associated with hemorrhage, is thought to be the primary defect and has been shown to induce a variety of alterations in cellular functions. The bone marrow (BM) is the major source of immune cells in the adult and, elucidating its response in hemorrhagic shock will add to the mechanisms of its associated immune alterations. We previously reported BM failure after hemorrhagic shock [5, 13]. Furthermore, the presence of gut bacteria and bacterial translocation does not appear to influence myelopoietic suppression following hemorrhagic shock [6].

Blood withdrawal and replacement are confounding factors in the ability to dissect the effects of hypoxia alone in immune dysfunction associated with hemor-

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rhagic shock. Standard models utilizing in vivo [14–16] and in vitro [17–21] hypoxia have been used as surrogates for hemorrhagic shock and allow the contribution of hypoxia alone to be determined. Previously we observed hematopoietic alterations following hypoxia that were consistent with similar studies in rats subjected to hemorrhagic shock [5, 6]. Comparable levels of circulating cytokines were found in mice subjected to either hypoxia or hemorrhagic shock [22]. Furthermore, depressed hepatocellular and macrophage function is reported in rats exposed to either hypoxic conditions or hemorrhagic shock [16].

The in vitro evidence indicates that BM stroma exerts most of the hematopoietic alterations associated with hypoxia [17]. Since BM stromal cells are major support of hematopoiesis, we utilize an in vivo model to determine the significance of these findings. We examined the roles of specific BM cell subsets in myelopoietic responses to hypoxia. Time course studies indicate that hypoxia mediate initial stimulation of granulocytic-monocytic BM progenitors (CFU-GM) followed by inhibition at day 5. We determined whether IL-1, IL-6, and TGF-β induction in BM mononuclear cells (BMNCs) from hypoxic rats correlated with CFU-GM formation.

MATERIALS AND METHODS

Cytokines and Antibodies

Hoffman-La Roche (Nutley, NJ) provided recombinant human (rh) IL-1α. TGF-β, rabbit polyclonal anti-hTGF-β, and IL-1 receptor antagonist were purchased from R&D Systems (Minneapolis, MN). Murine anti-rat CD3 (IgM) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM were purchased from Caltag Laboratories (San Francisco, CA). Non-immune murine IgM was purchased from Sigma (St. Louis, MO).

Cell Lines

The TGF-β-sensitive cell line, Mink Lung Epithelial (CCL 64), was maintained as previously described [23]. Briefly, cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM glutamine (Cellgro, Mediatech, Herndon, VA), 15 mM HEPES (Cellgro, Mediatech), and 10% FCD (Hyclone, Logan, UT). D10.G4.1 and 7TD1, IL-1- and IL-6-dependent cell lines, respectively, were purchased from the American Type Culture Collection and maintained according to their instructions.

Rats

Female Sprague Dawley rats, 180–225 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed in the AAALAC-accredited Research Animal Facility at UMDNJ-New Jersey Medical School (Newark, NJ). Rats were allowed free access to food and water except during hypoxic exposure. The hypoxemia protocol was adapted from the model described by Chaudry et al. [24]. Two unrestrained rats were placed in sealed plastic chambers (32.4 × 16.5 × 17.1 cm) and then subjected to 1 hr of hypoxia by gas exchange through inlet and outlet ports. Gas mixture was 5% O_2 and 95% N_2 and flowed at a rate of 10 L/min. After hypoxic exposure, rats were returned to room air. The hypoxic rats had a PaO_2 of 30–35 mmHg, and their arterial pH fell from 7.41 ± 0.02 to 7.35 ± 0.03. Normoxic rats were also placed in parallel chambers for 1 hr without gas exchange. At days 1 and 5 after hypoxia, rats were anesthetized by intraperitoneal injection with 50 mg/kg ketamine and 15 mg/kg xylazine and then sacrificed by cervical dislocation.

Bone Marrow Clonogenic Assays

Total BM cells were flushed from rat femur with a 21-gauge needle attached to a syringe containing 5 ml RPMI 1640 (Life Technologies). Single cell suspension was prepared by flushing BM cells twice through the 21-gauge needle. Suspension cells were washed twice and then cultured for CFU-GM in clonogenic assays as described [13]. Briefly, 10^5 cells were cultured in quadruplicate at 37°C, 5% CO_2 in 1 mL of 0.3% agar prepared in McCoy's medium (Life Technologies). Cultures were supplemented with 10% (v/v) conditioned media and 10% (v/v) fetal calf sera (Life Technologies). Conditioned medium was prepared by stimulating rat spleens with Pokeweed (Sigma) as described [25]. At day 10, a single blinded observer (D.H.L.) enumerated colonies with greater than 50 cells.

Preparation of Stroma-Depleted BMNCs

BMNCs were separated from total BM cells by Ficoll Hypaque density gradient (Sigma). Stroma-depleted BM cells were performed by passing unfractionated BM cells through scrubbed nylon wool columns (Fenwall Laboratories, Deerfield, IL) as described [26]. Stroma-free fractions were collected in the eluate. The differences in CFU-GM before and after nylon wool passages were ±5%, $N = 6$. This is consistent with other reports that showed no significant change in $CD34^+$ population following nylon wool separation [26].

TGF-β Quantitation

Growth inhibition of CCL 64 cells forms the basis of the bioassay used to quantitate active TGF-β [23]. Each sample was tested in triplicate with 25, 50, or 100 μL of supernatants. TGF-β levels were determined from a standard curve established with TGF-β concentrations ranging from 0.001 to 10 ng/mL. Samples that contained >20 ng/mL bioactive TGF-β were repeated in the presence or absence of rabbit panspecific polyclonal anti-TGF-β.

IL-1 Determination

IL-1 bioactivities were determined in a proliferative assay as described [27]. The bioassay for IL-1 is based on the proliferative responses of concanavalin A (Sigma)-stimulated mouse helper T cell clone, D10.G4.1. Each assay included a standard curve that was established with serial dilutions of either rhIL-1α or rhIL-1β. The highest concentration of standard cytokine was 500 U/mL followed by eleven serial dilutions. One unit of IL-1 was assigned as the half-maximal tritiated thymidine ([^{3}H]TdR) in a standard curve established with IL-1 concentration versus [^{3}H]TdR incorporation.

IL-6 Determination

IL-6 bioactivity was quantitated as described [14]. Briefly, serially diluted supernatants were cultured for 72 h with 10^5 7TD1 cells in 96-well plates. Cell proliferation was based on the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) during 2 hr. Absorbance at 570 nm was used to determine dye uptake.

Immunofluorescence Assays

Cells were labeled for 30 min at 4°C with murine (IgM) anti-rat CD3. Cells were washed and then reincubated for 20 min at 4°C with FITC-conjugated mouse anti-rat IgM. Cells were washed, fixed in 1% paraformaldehyde, and then analyzed by flow cytometry (Becton Dickinson). The following controls were performed: (1) FITC-conjugated second antibody; (2) media alone; (3) non-immune murine IgM followed by incubation with FITC-conjugated second antibody.

Statistical Analysis

Data were analyzed using analysis of variance and Tukey–Kramer post hoc test. Significance was determined at $P < 0.05$.

RESULTS

Cellularity in Femurs of Normoxic and Hypoxic Rats

We determined the total number of viable cells/femur at 1 and 5 days after hypoxic exposure. Cell viability was determined by trypan blue exclusion. We observed no significant difference in the total number of cells between normoxic and hypoxic rats (Table I). The percentage viability was 99% in all groups. The data show no evidence of necrosis or altered cellularity in the femurs of rats exposed to hypoxia.

TABLE I. Total Viable Cells per Femur From Normoxic and Hypoxic Rats[a]

Groups	Total number of cells × 10^7 ($\bar{x}$ ± SD)
Normoxic	9.5 ± 1.2
1 day post-hypoxia	8.1 ± 1.1
5 day post-hypoxia	10.2 ± 1.8

[a]Rats were subjected to hypoxia for 1 hr. At 1 and 5 days post-hypoxia, animals were sacrificed and the total cells per femur enumerated. Cell viability was consistently >99%. The results are expressed as the mean of six different experiments. In each experiment, three rats comprised one group.

Effects of Hypoxia on CFU-GM

Compared to normoxic rats, CFU-GM was significantly ($P < 0.5$) increased after 1 day with a downward trend toward baseline by day 5. The results of six different experiments, three rats per group, are shown in Figure 1. The results indicate that hypoxia initiates a significant stimulatory effect on CFU-GM. This effect on CFU-GM was attenuated by day 5.

CFU-GM in Stroma-Depleted BMNC From Hypoxic and Normoxic Rats

BM stromal cells are major cellular support of hematopoiesis under normal condition [28] and have been implicated in BM dysfunction following hypoxia [17]. Stromal depletions were accomplished by passage of BMNC through nylon wool columns. In normoxic rats, CFU-GM colonies in cultures with unfractionated and stroma-depleted cells were not statistically significant (Fig. 1, $P > 0.5$). However, in hypoxic rats, stroma-depletion caused significant decrease in CFU-GM (unfractionated, 138 ± 12; stroma-depletion, 48 ± 5).

The next set of experiments was designed to determine if a change in percentage of T-cells could account for the above results, as T-cells are a rich source of positive and negative regulators of hematopoiesis [29]. Since T-cells do not adhere to nylon wool, the ratio of T-cells to hematopoietic progenitors is expected to increase after nylon-wool passage. We determined the percentages of T-cells before and after nylon-wool separation from three randomly selected experiments. Cells from unfractionated and stroma-depleted samples were labeled with anti-CD3 and then analyzed by flow cytometry. The results showed no significant difference in the percentage of T-cells between the two cell populations: nylon-wool passage, 6.6 ± 1.4, versus unfractionated, 7.9 ± 2.7 (±SD). The results indicate that the number of T-cells within the two BMNC populations are similar and may not account for the differences in colony formation. Taken together, the results presented in this section suggest a major role for BM stromal cells in the stimulation of CFU-GM in hypoxic rats.

Effects of Hypoxia on IL-1, IL-6, and TGF-β Production in BMNC

IL-1 indirectly regulates hematopoiesis through induction of other cytokines [30]. In addition, IL-6 synergizes

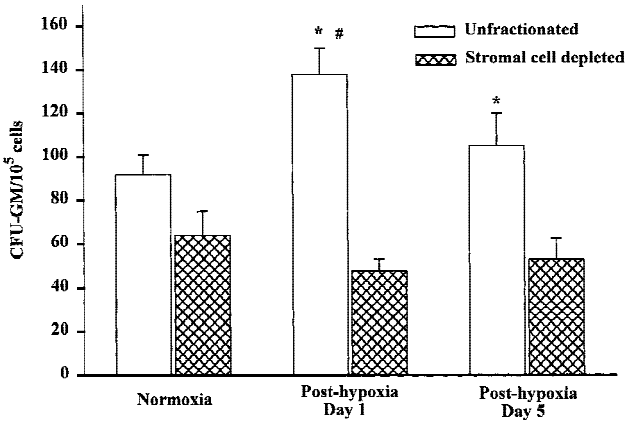

Fig. 1. CFU-GM formation in normoxic and hypoxic BMNC with and without stromal cells. Rats were subjected to hypoxia and at 1 and 5 days following hypoxia, either unfractionated or stroma-depleted BMNCs were cultured for CFU-GM. Parallel assays were performed with cells from normoxic rats. Each experimental point is the mean (±SD) of 18 cultures that include six different experiments with three rats per group. Details of the techniques are described in Materials and Methods. *$P < 0.05$: vs stroma-depleted cells on same day. #$P < 0.05$ normoxia.

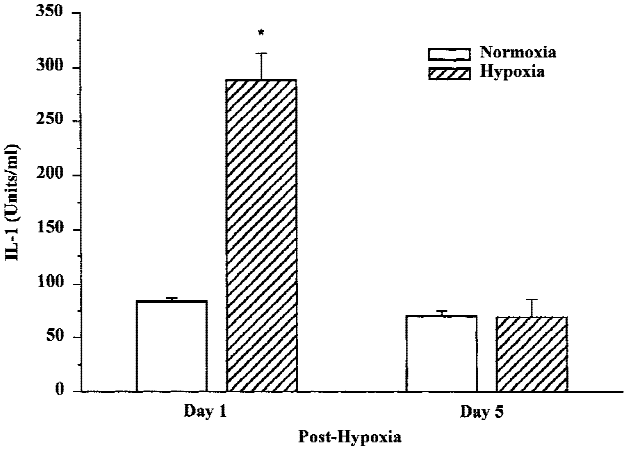

Fig. 2. Bioactive IL-1 produced by BMNCs from day 1 and day 5 post-hypoxia. BMNCs from rats that were either normoxic or day 1 and day 5 post-hypoxia were cultured for 24 hr in RPMI 1640 and 10% FCS. Cells-free supernatants were assayed for bioactive IL-1 in a proliferative assay with the D10.G4.1 cell line. The techniques are described in Materials and Methods. *$P < 0.05$ versus day 1 normoxia and day 5 hypoxia.

with other growth factors in the proliferation and differentiation of BM stem cells [31]. Cytokine levels were quantitated in the supernatants of BMNC obtained at days 1 and 5 post-hypoxia. Bioactive IL-1 was 3.5-fold more in the supernatants obtained in cultures with day 1 post-hypoxic cells (Fig. 2). By day 5, IL-1 levels were similar to normoxic cultures. The reduction in IL-1 levels by BMNC from day 5 post-hypoxia rats was significant ($P < 0.05$) compared to day 1 post-hypoxia. Bioactive IL-6 levels in day 1 and 5 post-hypoxic rats were significantly ($P < 0.05$) reduced by 4-fold when compared to normoxic controls (Fig. 3).

TGF-β inhibits cycling of stem cells and exerts a blunted effect on committed BM progenitors [32, 33]. Since hypoxia mediates an initial up-regulation of CFU-GM at day 1 and a decrease at day 5 (Fig. 1), we next determine levels of active TGF-β in the supernatants of day 1, 2, and 5 post-hypoxic BMNC. Comparison was made with normoxic rats. The results showed minimum increase at day 2 and significant increase at day 5 (Fig. 4). TGF-β activities were neutralized with the panspecific antibody. Titration assays indicate that 1 ng of antibody neutralize the bioactivity of 1–10 ng/mL in the supernatants. Compared to normoxic rats, hypoxic BMNC showed changes in their ability to produce IL-1, IL-6, and TGF-β. At day 1, IL-1, and IL-6 levels were increased and decreased respectively. At day 5, IL-1 levels were returned to baseline and IL-6 levels were unchanged. The data also show that TGF-β was down regulated at days 1 and 2 and up regulated at day 5.

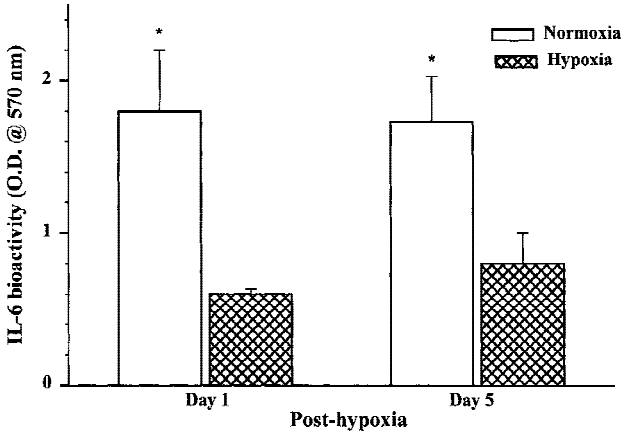

Fig. 3. Bioactive IL-6 produced by BMNCs from day 1 and day 5 post-hypoxic rats. BMNCs from rats subjected to hypoxic exposure after 1 and 5 days were cultured for 24 hr in RPMI 1640 and 10% FCS. Cell-free supernatants were assayed for IL-6 using the 7TD1 cells. The techniques are described in Materials and Methods. *$P < 0.05$ versus hypoxia on same day.

DISCUSSION

In this study, we showed that hypoxia mediates significant stimulatory effects on CFU-GM. In the absence of another biological insult, this stimulation reverts toward homeostasis (Fig. 1). Changes in BM cellularity or necrosis (Table I) could not explain the initial increase in CFU-GM progenitors in the hypoxic rats (Fig. 1). Clonogenic assays with stroma-depleted BMNC from hypoxic rats blunted the increase in CFU-GM (Fig. 1). In contrast to hypoxic rats, CFU-GM in stroma-containing

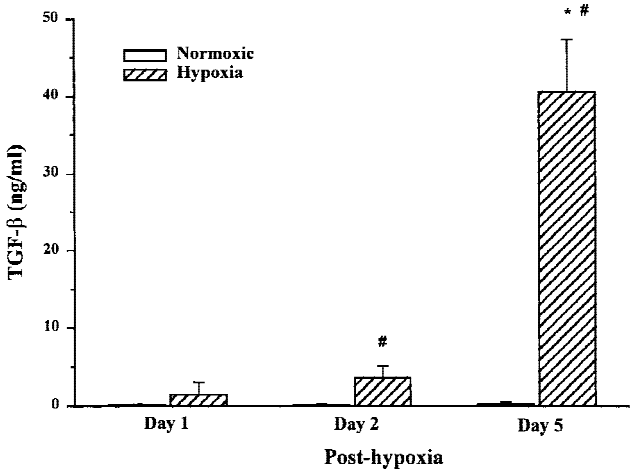

Fig. 4. Active TGF-β levels in the supernatants of BMNCs from day 1 and day 5 post-hypoxia. BMNCs from rats subjected to hypoxic exposure after 1 and 5 days were cultured for 24 hr in RPMI 1640 and 10% FCS. Cells-free supernatants were assayed for active TGF-β with the CCL-64 cells. Day 5 supernatants were assayed in parallel in the presence of anti-TGF-β. The techniques are described in Materials and Methods. *P* < 0.05 versus hypoxia days 1 and 2. #*P* < 0.05 versus same day normoxia.

and stroma-depleted cells were not significantly different in normoxic rats (Fig. 1). These results suggest that hypoxia may cause a putative response in BM stromal cells, which could lead to proliferation of CFU-GM progenitors. These results are consistent with a recent report that shows that hypoxia induces stimulatory hematopoietic growth factors in human BM stroma [17, 18]. Studies are underway in our laboratory to determine whether BM stroma affects CFU-GM stimulation through direct or indirect mechanisms. Although phenotypic analyses showed no change in the percentage of T-cells before and after nylon wool, a role for T-cells in hypoxia is yet to be determined. Furthermore, although we showed no difference in total BM cellularity between normoxic and hypoxic rats, future studies will investigate if there is a change in the distribution of progenitors and differentiated cells.

A complex network among cytokines, neurotrophic factors, neuropeptides and neurotransmitters [reviewed in Ref. 34] maintains hematopoietic regulation. Indeed, this study reinforces the role of cytokines in hypoxia-mediated effects on hematopoiesis. Hypoxia enhanced IL-1 production (Fig. 2). Maximum IL-1 induction corresponds to increased CFU-GM (Fig. 1). Compared to this report where we showed an increase in IL-1 (Fig. 2), we previously showed no evidence for IL-1 translation despite an increase in its mRNA [14]. Since our previous model used rats, this seemingly contradiction may be attributed to differences in species. The mechanism in which IL-1 is involved in the initial stimulation of myelopoiesis is unclear. We reported that hypoxia induces relevant hematopoietic growth factors, c-kit ligand and substance P, in BM stroma [17, 18]. Since IL-1 can also induce these two cytokines in BM stroma [34, 35], it is tempting to speculate that IL-1 may be a necessary cytokine in hypoxia-mediated hematopoietic effects. In addition, IL-1 has been reported to protect stem cells after BM insult from radiation and chemotherapy [36, 37]. This role of IL-1 may be relevant in BM insult secondary to hypoxia caused by hemorrhagic shock. Physiological systems tend to have a mechanism of feedback and hematopoietic stimulation by hypoxic exposure is no exception. TGF-β production in BMNC is correlated with CFU-GM in hypoxic rats (Figs. 1 and 4). Since this cytokine is associated with negative hematopoietic regulation, its induction could be part of the feedback process to attain hematopoietic homeostasis. A role for TGF-β as a feedback in hypoxia-mediated stimulation of CFU-GM is a current subject of investigation in our laboratory with human BM cells. At present, we have no explanation for the reduced levels of IL-6 in rats subjected to hypoxia. In murine, IL-6 exerts direct stimulatory effects on granulocytic progenitor [38], and our studies with human cells indicate that IL-6 exerts suppressive effects on CFU-GM during hypoxia (unpublished). Low levels of IL-6 produced in BMNC from hypoxic rats could be due to a species-specific effect on CFU-GM.

The initial stimulation of CFU-GM at day 1 followed by a decrease by day 5 may partly explain the bimodal immune responses that are reported following shock [reviewed in Ref. 39]. A trend toward baseline by day 5 post-hypoxia may be due to the lack of another insult that would exacerbate immune dysfunction and lead to organ failure, the "two-hit hypothesis" of multiple organ failure [3, 40]. These studies dissect the effects of hypoxia alone in hematopoiesis and could provide an understanding of later complications in hemorrhagic shock such as those mediated by other organ dysfunction. These understandings could lead to strategies for therapeutic interventions during the early stages following hemorrhagic shock.

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